Effect of red mud on the microbial activity of sandy soils
OTKA PD 115871 project, 2016.01.01.–2017.12.31., final report
Made by Dr. Viktória Feigl

1. Project background

The aim of the project was to investigate the effect of low amounts (≤10 w/w%) of red mud (bauxite residue, waste from the alumina production from bauxite by the Bayer process) on the microbial activity of sandy soils. 150–170 million tonnes of this waste material is produced every year, so re-use options are needed (Evans, 2016; Hua *et al.*, 2017). Previous studies were focusing mostly on the physical and chemical effects of red mud in sandy soils when applied as soil amendment (Summers *et al.*, 1993; Snars *et al*., 2004), however, the effect on the soil microbial community was less studied, only in some cases when red mud was used as chemical stabilizer for metal contaminated soils (Lombi *et al*., 2002; Garau *et al*., 2007; Castaldi *et al*., 2009). In this project we were focusing on uncontaminated sandy soils with little or no nutrient content, where red mud can improve water and nutrient holding capacity by improving soil structure (Barrow *et al.*, 1982; McPharlin *et al.*, 1994).

2. Project tasks

The project was divided into seven main tasks and three project milestones. First, we carried out a detailed literature survey, then we measured the physical-chemical-microbiological-ecotoxicological properties of Hungarian red muds and sandy soils. Soil microbial assessment methods were adjusted to the problem of red mud amended soils. In the second year a soil microcosm experiment was carried out and monitored for 7 months. The results were evaluated by statistical methods.

3. Project results

3.1 Literature survey

The extended literature survey (only the most important points are highlighted in this report) revealed, that although red mud is a harsh environment, it contains its own microflora (Agnew *et al.*, 1995; Krishna *et al.*, 2014). These alkalophilic and haloalkaliphilic microorganisms can be used in various biotechnological applications (Horikoshi, 1999), e.g. for the bioremediation of red mud deposits (Santini *et al.*, 2015). Red mud, when mixed into soils as re-use option or accidentally, has effects on the soil's physical, chemical, ecotoxicological and microbiological properties (Ruyters *et al*., 2011; Rékási *et al*., 2013; Alshaal *et al*., 2014, Mayes *et al*., 2016). Previous studies suggested, that red mud can be mixed into the soil at up to 5 w/w% (Ujaczki *et al.*, 2015, 2016).

3.2 Microbial community in Hungarian red muds

We have made the first attempt (based on literature) to investigate the microbial community in Hungarian red muds. Three red muds (RM) were chosen: RMA – "fresh" red mud, filtered, gypsum treated, samples from 2016 (pH=8.8, EC=1.2 mS/cm, dry matter content=80%), RMB

– stored red mud, samples from 2016 (pH=8.9, EC=0.9 mS/cm, dry matter content=67%), RMC – stored red mud, samples from 2014 (pH=9.1, EC=0.7 mS/cm, dry matter content=74%).

Microbial strains were isolated on alkaline nutrient, malt and Horikoshi agar plates (pH=9) (Krishna *et al*., 2014), and a strain collection was created. The 16S rRNA gene of bacterial strains was amplified with universal 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') primers (Lane, 1991), while the internal transcribed spacer (ITS) regions of fungal ribosomal DNA were amplified with ITS1-F (5' CTT GGT CAT TTA GAG GAA GTA A 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') primers (Gardes *et al.*, 1991). Based on the sequence similarity the bacterial identifications were carried out by EZTaxon, while fungal identifications by UNITE and NCBI, GenBank databases (Török, 2017). The pH (5–10), salt (0–15%) and temperature (5–50 °C) tolerance was measured in modified nutrient media. The siderophore (small iron chelating compound) and extracellular polymeric substance (EPS) production capacity of the isolates was assessed (Kutasi *et al*., 2015; Bombolya, 2017). Nitrogen-fixing, phosphorous mobilizing and cellulose degrading potential was investigated on Ashby, Pikovskaya, and Fuller-Norman agar plates.

At least 35 bacteria and 8 fungi species were isolated from the red mud. The number of microbes was $3 \cdot 10^6$ CFU/g dry red mud in nutrient agar, $3 \cdot 10^3$ CFU/g dry red mud in malt agar, and $5 \cdot 10^5$ CFU/g dry red mud in Horikoshi agar. 20 strains were identified at genus or species level. Bacteria species included *Bacillus aryabhattai*, *Bacillus beringensis*, *Bacillus coreaensis*, *Bacillus pseudofirmus*, *Brevundimonas diminuta*, *Nesterenkonia massiliensis*, *Massilia timonae*, *Ochrobactrum pseudogrignonense* and *Pseudomonas stutzeri*. Fungi species were identified as *Aspergillus* and *Penicillium* strains. One strain was *Aspergillus sydowii*. Most of them are species able to colonize a wide variety of habitats, such as soils, cold seas, or highly alkaline lakes (Török, 2017, Feigl *et al.*, 2016, 2017a).

Most of the isolates tolerated the entire investigated pH range (pH=5–10), and for many the optimal pH for growth was at 8–9, while six strains showed the highest growth at pH 10. Salt tolerance was investigated at up to 15% NaCl in the media, and six strains were able to grow at this high salt content. Most of the strains tolerated salt content at up to 5–7%. The ideal temperature for growth was 20–40 °C for most bacteria and fungi, but some were able to grow at 5 °C, or at 50 °C. Most of the strains produced extracellular polymeric substances and siderophores. Half of the stains were able to mobilize phosphorous and around 70% were cellulose degraders. Some nitrogen-fixing bacteria were also found (Bombolya, 2017, Feigl *et al.*, 2017a).

The first milestone of the project "Determination of the activity of the microbial community and of the physical-chemical characteristics of the red mud and soils" was reached and the first key question / hypothesis "Does the Hungarian red mud contain an own microflora and how active is it?" was answered to.

3.3 Microbial assessment methods for red mud amended soils

Soil microbial assessment methods were chosen based on infrastructural availability and literature. Newly introduced methodologies, that had not been used previously routinely in the BME lab, were the following:

- Living cell number measurements for various microbial groups based on plate count techniques and MPN methods. Copiotrophic microorganisms favour nutrient rich conditions, while oligotrophes can handle nutrient scarcity and develop communities at these circumstances (Krzyżak *et al.* 2013). We used and developed two methods based on plate count techniques for the determination of oligotrophic aerobic heterotrophic cell numbers (Szaszák, 2016). Nitrogen-fixing (Asby-agar), phosphate mobilizing (Pikovskaya-agar) and Actinomycetes (Kenknight-agar) living cell numbers were also measured (Berkl, 2018).

- Substrate induced respiration can be measured in closed bottle test in OxiTop system. The methodology allows respiration measurements in soils with originally low respiration without substrate addition (Szaszák, 2016; Tóth, 2016; Major, 2017a,b).

- Soil enzyme activity measurements can be applied for the assessment of the functional diversity of microbes in soil. Soil enzymes react quickly to environmental changes (Alef and Nannipieri, 1998). They play an important role in the biogeochemical cycling of elements (Das and Varma, 2011). β-glucosidase (C-cycle), acidic, neutral and alkaline phospomonoesterase (P-cycle) and urease (N-cycle) enzyme activities were measured (Rottek, 2017; Dávid, 2018).

Some methods had to be adapted to the specific problem: the red colour of red mud and its small particle size. In the case of methodologies based on colour change (e.g. enzyme activity measurements) filtration through medium velocity (4–12 μm pore size) filters and centrifugation (at 10 000 rpm for 10 min) of soil extracts are needed (Dávid, 2018).

Our preliminary studies for the microbial activity assessment of red mud treated soils used BIOLOG EcoPlate method, which is based on the substrate utilization capacity of 31 substrates, and allows community-level physiological profiling (CLPP) of heterotrophic bacterial assemblages in the soil (Insam, 1997). We showed, that the various evaluation parameters, such as Average Well Colour Development (AWCD), substrate richness (SR) and Shannon diversity index (H%) can be used for the follow up of the microbial effects of red mud in soil. We showed that red mud at up to 20 w/w% in acidic sandy soil increased AWCD on the short-term, but this effect did not last for 10 months. Microbial diversity in the treated soils also decreased during this period (Feigl *et al*., 2016, 2017b).

The second milestone of the project “Development of specific microbial activity measurement methodologies to the red mud containing soils” was reached.

3.4 Investigation of the effect of red mud on soil microbial activity in a 7 months microcosm experiment

In a microcosm experiment Hungarian red mud (RM, pH=8.9, dry matter content: 66%, total metal content under the Hungarian Quality Criteria for sewage sludge from waste water treatment for agricultural applications based on Government Decree No. 40/2008) originating from a storage facility was mixed into two sandy soils from Hungary: an acidic sandy soil (ASS from Nyírlugos, pH=4.9, sand=85%) and a carbonated sandy soil (CSS from Őrbottyán, pH=8.2, sand=81%). Red mud is usually used for the improvement of acidic soils (Summers *et al.*, 1993; Snars *et al.*, 2004), while the carbonated sandy soil option studied the case where possibly the pH changing effect of red mud is not the main effect. Red mud was mixed into the soils at 0, 1, 2,5; 5; 7,5; 10 w/w% in 3 kg microcosms with 3 replicates. We elected to use the above RM concentration range since in our previous studies the effect of lower than 5 w/w% RM had not been tested (Ujaczki *et al.*, 2015, 2016), and the 10 w/w% RM proved to have still microbial activity enhancing effect in the soil (Feigl *et al.*, 2016, 2017b). The microcosms were watered every 2nd week to 60% water holding capacity, and kept at room temperature (22±2 °C) hidden from light. Microcosms were sampled at the beginning of the experiment (instant effects), at 2 months (short-term effects) and at 7 months (mid-term effects).

The integrated monitoring methodology combined physical, chemical, biological and ecotoxicological methods. Ecotoxicity assessment was carried out in the frame of OTKA PD 121172 project (we do not detail results here). Water holding capacity (WHC, Buzas, 1993), pH, electrical conductivity (EC), water content (MSZ 21470/2-81, 1982) and ignition loss (Sluiter *et al.*, 2008) was measured at BME. Total (Aqua Regia extract) and water soluble (distilled water extract) content of Al, As, Co, Cr, Cu, Mo, Na, Ni, V, Zn was measured according to MSZ 21470-50:2006 by ICP-OES. The monitored metals were chosen based on previous experiments (Ujaczki *et al.*, 2015, 2016). Plant available (ammonium-lactate extractable) P and K content (MSZ 21470-50:2006), NO_3^--N, NH_4^+-N (MSZ 20135:1999), total N (MSZ-08-0012-10:1987) and humus (Tyurin, 1931) content was measured as background parameters. These were measured at the Institute for Soil Sciences and Agricultural Chemistry, Centre for Agricultural Research, Hungarian Academy of Sciences. At the Bay Zoltán Nonprofit Ltd. for Applied Research we investigated the particle size distribution of the treated soils by Keyence VHX-2000 HDR Multiscan Digital Microscope.

The main aim of the project was focusing on the microbiological effects of red mud in soils. Living cell number determination included aerobic heterotrophic bacteria, fungi, Actinomycetes, phosphorous mobilizing bacteria, nitrogen-fixing bacteria; copiotrophic and oligotrophic cell number by plate count technique, and aerobic and anaerobic heterotrophic bacteria number by Most Probable Number method. Soil enzyme activities included dehydrogenase (for overall oxidative microbial activity), β-glucosidase, acidic, neutral and alkaline phosphomonoesterase and urease. Substrate induced respiration measurements and community-level physiological profiling by BIOLOG EcoPlate were carried out. (For references see chapter 3.3.)

Statistical analysis was carried out to compare the effects of different red mud amounts in soils by StatSoft® Statistica 7. Analysis of variance (one-way ANOVA), Fisher LSD or Neuman-Keuls comparison were used ($p<0.05$). Pearson Product Moment Correlation Analysis was performed, correlation was considered strong when the correlation coefficient (r) was higher than 0.60 and very strong at $r \geq 0.85$.

As the monitoring methodology applied for the follow-up of the effect of red mud in soils was very complex and included 3 samplings, only the main results are presented in this report.

The **pH** of both soils significantly increased even at 1% RM addition compared to the control. For example, in the case of ASS (pH=4.9, acidic) 1% RM addition resulted in pH=7.3 (neutral pH according to Stefanovits *et al.*, 1999), 5% RM in pH=8.6 and 10% RM in pH=9.5. ASS soil became alkaline (pH>8.5) at 5% RM, while the slightly alkaline CSS soil (pH=8.2) became alkaline at 1–2.5% RM amount. **Plant available P and K** increased with increasing RM dose in both soils.

The total and water extractable metal and metalloid content in the RM treated soils was compared to the Hungarian Quality Criteria (QC) for soil and groundwater, respectively, based on KvVM-EüM-FVM Joint Decree No. 6/2009. For Al, Na and V there is no Hungarian QC, so we compared these values to groundwater QC in different EU states (European Commission, 2010), Ajka specific QC (Gruiz *et al.*, 2013) and Dutch risked based QC (Swartjes, 1999). In summary, most metals fulfilled the limit values in soils at up to 10% RM, except for mobile As, Mo and V. All three metals exceeded QC for subsurface waters after 7 months at 7.5–10% RM dose in both soils, while As exceeded QC in 5% RM treated soils at 2 months, but was under QC at month 7. Total As content exceeded Hungarian QC at 7.5–10% RM, total Na was above the chosen quality criteria at 2.5% and higher RM doses, but the mobile Na content of soils was under European QC. This means, that based on metal content, maximum 5% RM amount may be allowed, in accordance with previous studies (Ujaczki *et al.*, 2015, 2016).

The aerobic heterotrophic living cell numbers increased significantly after 7 months at 2.5–10% RM addition to ASS compared to the control, but in CSS only at 10% RM. Instant effect of RM caused the increase in copiotrophic cell numbers (from 33% to 55% at 2.5% RM dose) possibly due to the addition of nutrients with RM to the soil, however, after 7 months, the ratio of oligotrophic cells was around 60% in all treated ASS, meaning the development of a stabile microbial community able to live under nutrient deficient conditions (Krzyżak *et al.*, 2013). **Other microbial cell counts** usually increased in ASS upon 5–10% RM, and in CSS upon 7.5–10% RM addition. **Oligotrophic aerobic living cell numbers** in ASS determined on oligotrophic media increased with incremental RM amount from 2.5% RM dose (4.2 times increase at 10% RM). In CSS oligotrophic cell numbers increased at 5–10% RM dose (14 times increase at 10% RM). **Anaerobic cell numbers** significantly increased at 2.5–10% RM in ASS (>1000 times increase), and at 10% RM in CSS. Usually, there was a strong positive correlation between the living cell numbers and the RM dose, pH, ignition loss, available P and K. In summary, if we only consider living cell numbers determined by plate count techniques, which represent the active and potentially active microbial population in soils highly contributing to

nutrient cycling (Blagodatskaya *et al*., 2013), 2.5–10% RM addition in ASS, 7.5–10% RM addition in CSS could be favourable from microbial point of view (Berkl, 2018).

Dehydrogenase enzyme activity (DEH, oxidative microbial activity) significantly increased with RM addition in ASS even at 1% dose (5.5 times increase at 10% RM) compared to the control, while in CSS only 10% RM resulted in significant increase (1.1 times) after 7 months. **Substrate** (glucose) **induced respiration** (SIR, aerobic heterotrophic activity) increased 1.5 times at 1–7.5% RM dose in ASS, but decreased to the half at 10% RM after 7 months. In CSS RM addition caused a decrease in SIR even at 1% RM, the highest decrease was 0.6–0.7 times at 7,5–10% RM. **The AWCD in BIOLOG EcoPlate** showed similar patterns to SIR: ~2 times increase at 1–7.5% RM dose and no change at 10% RM after 7 months in ASS. Although AWCD was high due to 5–10% RM addition after 2 months (max. 5 times increase), this increasing effect of RM did not last for 7 months. Similar pattern was found during preliminary studies (Feigl *et al*., 2017). **Substrate richness** (SR) also increased 2 times by RM addition at 1–10% in ASS after 2 months, but remained significant only at 1–2.5% RM in treated ASS (1.4 times increase) after 7 months. **Shannon diversity** (H%) decreased significantly (0.8 times) in 7.5–10% RM treated ASS after 7 months. In CSS RM starting from 2.5% dose caused significant decrease in AWCD, SAWCD, SR and H%. Most of the chemical parameters correlated with the above detailed microbiological parameters right after RM addition to soils, but after 7 months, only the pH was correlating positively with DEH, while pH and RM dose correlated negatively with SIR and AWCD. To sum up, based on SIR and BIOLOG EcoPlate results, the microbial activity enhancing effect of RM only lasts at small RM amounts (1–2.5%) in ASS, but in CSS the effect of RM is negative from 2.5% RM even on the short term (Major, 2017a,b).

The **β-glucosidase** activity significantly decreased in both soils from 1% RM addition with the increasing RM dose. In ASS acidic and neutral **phosphomonoesterase** (PME) decreased significantly with RM addition, while alkaline PME increased. However, if we sum up all PMEs, PME activity is decreasing with incremental RM amount, except for 10% RM, but their activity is still 2/3 of the untreated control. PME in CSS was not influenced by RM addition. **Urease** activity significantly increased at 7.5–10% RM in ASS, and at 2.5–10% RM in CSS after 7 months. In ASS enzyme activities negatively correlated with RM dose, pH, available P and K content (Dávid, 1018).

Based on the above presented selected results it can be concluded that RM addition influenced the chemical composition of the sandy soils by increasing soil pH and mobile ion content, including both nutrients (P, K) and toxic metals and metalloids. Red mud increased living cell numbers and overall oxidative microbial activity (DEH) in both soils, and the highest increase was observed at the highest red mud doses: 7.5% and 10%. However, SIR, BIOLOG EcoPlate and enzyme activity measurements make the picture more complicated showing the most negative effects at the highest RM doses in some cases.

As careful recommendation, red mud addition can be used in acidic sandy soils at up to 5 w/w%, the optimal dose is ~2.5% red mud. Red mud treatment is not recommended in carbonated sandy soils.

The third milestone of the project "Evaluation of the short and medium term sampling and monitoring results" was reached. The second and third key question / hypothesis "How does the microbial activity of the sandy soils change by the presence of red mud?" and How do the changes in the sandy soil's microbial activity correlate with other soil characteristics?" were answered to.

4. Conclusions

During the 2 years of this OTKA PD project a detailed picture was created about the microbial composition of Hungarian red mud, and the microbial effect of red mud treatment on sandy soils. All project milestones were reached, and the raised questions were answered to. Of course, in the case of red mud, longer (than 7 months), and more detailed (e.g. metagenomics) monitoring, or investigation at field conditions combined with the sowing of plants (to investigate rhizosphere effects and bioaccumulation) could be the next step to understand better the effects of this waste material on the soils, and to make decisions on the applicability of this re-use option.

5. Dissemination

The project results focusing on the BIOLOG EcoPlate methodology was published in the Science of the Total Environment (impact factor 4.9) (Feigl *et al*., 2017b). Two other journal articles evaluating previous studies related to the topic were also published in Journal of Chemical Technology & Biotechnology (impact factor: 3.135) and Journal of Sustainable Metallurgy (no impact factor, but 17 independent citations so far) (Ujaczki *et al*., 2016; Mayes *et al*., 2016). The results presented in Chapter 3.2 and 3.4 are planned to be published also in impact factor journals in two papers.

Project results were presented at three conferences: poster presentation at 10th ISEB Conference 2016, 1–3 June 2016, Barcelona, Spain and 16th Alps-Adria Workshop – Synergism in Science, 3–8 April 2017, Opatija, Croatia; oral presentation at 9th International Conference on Environmental Engineering and Management, 6–9 September 2017, Bologna, Italy (Feigl *et al.*, 2016, 2017a,c).

One BSc thesis (Major, 2017b) and seven MSc theses (Berkl, 2018, Bombolya, 2017, Dávid, 2018, Rottek, 2017, Szaszák, 2017, Tóth, 2017, Török, 2017) were dealing with the topic of this research. Major (2017a) won 2nd place at the Scientific Student Competition in the Faculty of Chemical Technology and Biotechnology of BME and the price of the American Society of Microbiology with her work.

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